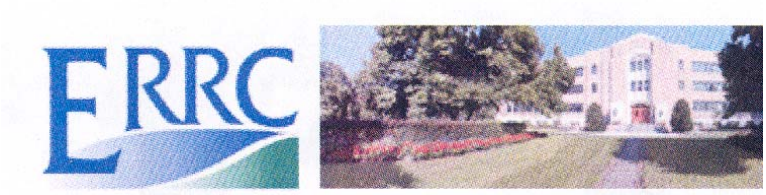

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(215) 233-6400

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Author(s): V.K. Juneja, J.S. Novak, L. Huang, and B.S. Eblen

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Increased thermotolerance of *Clostridium perfringens* spores following sublethal heat shock

Vijay K. Juneja [a,*], John S. Novak [a], Lihan Huang [a], Brian S. Eblen [b]

[a] *US Department of Agriculture, Agricultural Research Service, Eastern Regional Research Center, 600 East Mermaid Lane, Wyndmoor, Pennsylvania 19038, USA*[1]

[b] *Food and Drug Administration, Center for Food Safety and Applied Nutrition, 200 C. Street SW, Washington, DC 20250, USA*

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Abstract

Beef gravy samples inoculated with *Clostridium perfringens* spores were heat shocked at 75 °C for 20 min, and then thermotolerance at 100 °C was assessed using a submerged-coil heating apparatus. Survivors were enumerated on Shahidi Ferguson Perfringens agar. An association of the heat resistance with the origin of the *C. perfringens* could not be established due to significant variations in the heat resistance among strains. Interestingly, deviations from classical logarithmic linear declines in the log numbers with time were not observed in both control and heat shocked samples. *D*-values at 100 °C for *C. perfringens* spores ranged from 15.5 to 21.4 min. Heat shocked spores of 9 out of 10 strains had significantly higher ($p < 0.05$) *D*-values at 100 °C than unstressed spores. Proteins with epitopic and size similarity to *Escherichia coli* GroEL and *Bacillus subtilis* small acid-soluble protein, SspC, were present in spores. However, heat shock treated spores did not appear to significantly increase expression of these proteins. Acquired thermotolerance is of substantial practical importance to food processors and should provide useful information for designing thermal treatments to eliminate *C. perfringens* spores in ready-to-eat foods.

Keywords: *Clostridium perfringens*; Heat resistance; Thermotolerance; Heat shock

1. Introduction

Clostridium perfringens type A is an anaerobic, gram-positive, spore-forming, rod-shaped, non-motile bacterium. The organism continues to remain a major cause of foodborne illness and a concern to the food service industry world wide. It has been implicated in 248,520 cases of foodborne illnesses every year in the United States with 41 hospitalizations and seven deaths (Mead et al., 1999). In 1994, the total cost of illnesses due to *C. perfringens* was estimated at $123 million in the US (Anonymous, 1995). The food poisoning results from the ingestion of a large number of viable vegetative cells of the organism in temperature-abused foods. The heat labile enterotoxin synthesized by sporulating cells in the small intestine is responsible for the pathological effects in humans as well as the typical symptoms of diarrhea and abdominal pain.

C. perfringens is found in soil, water, air, intestinal tract, and a variety of raw and processed foods, particularly meat and poultry. Because of the ubiquitous distribution, it is difficult if not impossible to exclude spores of this pathogen during the processing of various animal or plant products and its presence must be assumed. Therefore, the thermal treatment applied to processed foods must be adequate to destroy the spores or their germination, outgrowth and subsequent vegetative growth must be restricted, if the food is to be safe.

Heat treatment designed to achieve a specific lethality for foodborne pathogens is a critical control point in food processing and is fundamentally important to assure the shelf-life and microbiological safety of thermally processed foods. A key to optimization of the heating step is defining the target pathogen's heat resistance. The heat resistance of any given microorganism is known to be affected not only by inherent genetic factors, but also by the environmental factors

* Corresponding author. Tel.: +1-215-233-6500; fax: +1-215-233-6559.

E-mail address: vjuneja@arserrc.gov (V.K. Juneja).

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encountered prior to and during the cooking step. Adaptation to environmental stresses has received recent attention because of its implications in the safety of milder preservation technologies. Stresses such as storage and holding temperatures, low temperature-long time cooking expose the contaminating vegetative and spore-forming foodborne pathogens to conditions similar to heat shock, thereby rendering the heat shocked organisms more resistant to subsequent lethal heat treatments (Juneja, Klein, & Marmer, 1997). Accordingly, the work reported herein was undertaken to quantify the effect of heat shocking *C. perfringens* spores on their thermotolerance and compare the levels of select heat shock proteins. The quantitative assessment of heat resistance, the heat shock response and the induced thermotolerance, should assist food processors in designing thermal processes for the inactivation of *C. perfringens* spores, thereby ensuring the microbiological safety of cooked foods.

2. Materials and methods

2.1. Test organisms and spore production

Ten strains of *C. perfringens* originating from food poisoning, veterinary or human clinical cases, were used in the study (Table 1). The strains were kindly provided by Dr. Bruce McClane (University of Pittsburgh School of Medicine, Pittsburgh, PA). Stock cultures were maintained at 4 °C in cooked-meat medium (Difco Laboratories, Detroit, MI). Rabbit antiserum raised against *B. subtilis* small acid-soluble protein, SspC, was provided as a generous gift from Dr. Peter Setlow at the University of Connecticut (Framingham, CT). Rabbit polyclonal antibodies raised against *E. coli* chaperonin, GroEL, were purchased from StressGen Biotech. Corp. (Victoria, BC, Canada). An active culture was prepared in freshly prepared fluid thioglycollate medium, and sporulation was carried out in Duncan and Strong medium as previously described (Juneja, Call, & Miller, 1993). After the spore crop of each strain had been washed twice and resuspended in sterile distilled water, the spore suspensions were stored at 4 °C.

2.2. Spore heat resistance and data processing

The formulation of the beef gravy used as heating menstruum was the same as described previously (Juneja et al., 1997). Both heat shocked (75 °C/20 min) and non-heat shocked samples (controls) of beef gravy inoculated with *C. perfringens* spores were heated at 100 °C using a submerged-coil heating apparatus according to the procedure described elsewhere (Cole & Jones, 1990; Juneja et al., 1997). Samples were removed at predetermined time intervals and rapidly cooled in melting ice. The surviving population of spores were determined by spiral plating (Model D, Spiral systems, Cincinnati, OH) on Shahidi Ferguson perfringens agar as described earlier (Juneja, Marmer, & Call, 1996). The *D*-values (time for a 10-fold reduction in viable spores) were determined by plotting the log of survival counts versus heating time using Lotus 1-2-3 Software (Lotus Development Corporation, Cambridge, MA, USA) and taking the absolute value of the inverse slope.

The heat resistance data were analyzed by analysis of variance (ANOVA) using SAS (SAS, 1989) to determine if there were statistically significant differences among the treatments. Bonferroni mean separation test was used to determine significant differences ($p < 0.05$) among means (Miller, 1981). The data was also analyzed by analysis of covariance to determine the effect of

Table 1
The effect of prior exposure of *C. perfringens* spores in beef gravy at 75 °C for 20 min (heat shock) on the microorganisms heat resistance (expressed as *D*-value in min)[a] at 100 °C

Isolate	Origin	*D*-value (r^2)[b]	
		No heat shock	Heat shock
NCTC 8238	1950s European food-poisoning	16.65 ± 0.09 (0.95)^{ab}	18.40 ± 0.08 (0.95)^{e}
NCTC 8239	1950s European food-poisoning	15.50 ± 0.24 (0.97)^{b}	20.61 ± 1.00 (0.98)^{cde}
NCTC 10239	1950s European food-poisoning	20.17 ± 0.06 (0.95)^{ab}	26.54 ± 1.64 (0.96)^{ab}
153	1990s North American veterinary	18.41 ± 0.51 (0.98)^{ab}	26.02 ± 0.67 (0.98)^{abc}
222	1990s North American veterinary	15.84 ± 0.51 (0.95)^{b}	19.66 ± 0.85 (0.98)^{de}
FD 1041	1980s North American food-poisoning	18.28 ± 1.76 (0.97)^{ab}	25.96 ± 1.36 (0.92)^{abc}
C-1841	1980s North American food-poisoning	18.01 ± 0.55 (0.92)^{ab}	21.96 ± 1.12 (0.96)^{bcde}
F 4969	1980s European: clinical	16.43 ± 0.85 (0.91)^{ab}	24.00 ± 2.13 (0.93)^{abcd}
NB 16	1980s European: clinical	21.40 ± 0.78 (0.95)^{a}	28.07 ± 0.08 (0.98)^{a}
B 40	1980s European: clinical	19.10 ± 1.08 (0.95)^{ab}	23.19 ± 0.25 (0.96)^{abcde}

a, b, c, d, e: values in the same column followed by the same alphabet are not significantly different ($p < 0.05$).

[a] *D*-values shown are the means of two replicate experiments, each performed in duplicate and expressed as mean ± standard deviation. Heat shock resulted in significant increase ($p < 0.05$) in *D*-values of all isolates except NCTC 8238.

[b] Correlation coefficients in parenthesis.

treatment (heat shock versus non-heat shock) and strain on the slopes of the survivor curves (SAS, 1989).

2.3. Spore lysate preparation and analysis of expressed proteins

Approximately 7 $\log_{10}$ spores were suspended in 1 ml of lysis buffer (Rheinberger, Geigenmuller, Wedde, & Nierhaus, 1988) and broken using a Vibracell model VC130 ultrasonic processor (Sonics & Materials, Inc., Newtown, CT) for 2 min on ice using a 1 s pulse and amplitude setting of 60. Spores were resuspended in gel loading buffer (Sambrook, Fritsch, & Maniatis, 1989), boiled for 5 min, and evaluated using Western immunoblot analyses as described previously (Novak & Tabita, 1999). Antibodies raised against specific proteins were diluted 1:3000 for α-SspC and 1:5000 for α-GroEL, respectively.

3. Results and discussion

The present study determined the extent to which a heat shock at 75 °C for 20 min results in increased *D*-values at 100 °C of *C. perfringens* spores suspended in beef gravy. Fig. 1 depicts typical examples of the survivor curves, i.e., the destruction of both heat shocked and non-heat shocked *C. perfringens* spores in beef gravy at 100 °C. For non-heat shocked samples of beef gravy heated at 100 °C, spores of *C. perfringens* decreased by 2.11 logs (4.85 $\log_{10}$ CFU/ml to 2.74 $\log_{10}$ CFU/ml) within 40 min and by 4.00 logs (0.85 $\log_{10}$ CFU/ml) at 80 min (Fig. 1). In comparison to the controls, heat shocking spores at 75 °C for 20 min in beef gravy prior to heating at 100 °C for 40 and 60 min resulted in 1.30 and 1.95 log reductions, respectively, in *C. perfringens* spores counts/ml, from an initial inoculum of 4.88 $\log_{10}$ CFU/ml, and the log destruction was 3.02 $\log_{10}$ CFU/ml at 80 min. Thus, heat shocked *C. perfringens* spores exhibited higher heat resistance and survived longer than non-heat shocked spores.

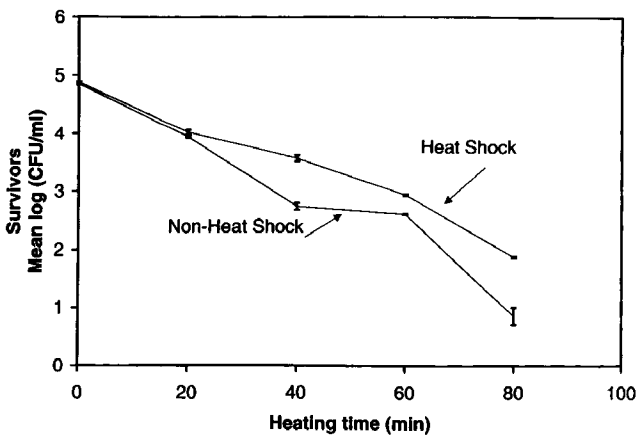

Fig. 1. Survivor curve of heat shocked (75 °C/20 min) and non-heat shocked *C. perfringens* spores (NB 16) in beef gravy at 100 °C.

In the present study, the slopes of the inactivation curves, as exhibited in Fig. 1, for both controls and heat shocked samples were linear, i.e., log number of survivors declined in a linear manner with time. Such linear survival curves suggest that all spores in the population had identical heat resistance. According to McKee and Gould (1988), it is merely the chance of a quantum of heat impacting a heat sensitive target in a cell or spore that determines the death rate. Our results are consistent with the universally accepted fact that the rate of destruction of spores followed first order kinetics, i.e., when a microbial population is heated at a specific temperature, the cells/spores die at a constant rate. This traditional first order kinetics model of thermal inactivation forms the basis of calculations used in thermal processing and has served the food industry and regulatory agencies for decades. This observation regarding the survivor curves exhibiting no apparent shoulders or tailing contradicts the reports and suggestions made by previous researchers working with vegetative cells and spores of foodborne pathogens (Juneja et al., 1995; Pagan, Condon, & Sala, 1997). Researchers have expressed concerns about the microbiological risk involved in the processing of new generation foods and indicated that deviations from linearity in the survivor curve are frequently observed when ready-to-eat products such as cook/chill, *sous vide* foods, etc. are processed (Juneja et al., 1997). Slow heating rate/long come-up times and low heating temperatures employed in the production of *sous-vide* cooked foods expose the microbial cells to conditions similar to heat shock, thereby rendering these cells more thermotolerant. Therefore, consideration of non-first order inactivation kinetics is important in the safe application of milder heat processes, though we, in the present study, did not observe significant and systematic deviations from classical logarithmic linear declines in the log number of survivors with time, even in the case of heat shocked samples.

It was not feasible to establish a relationship between the heat resistance of spores at 100 °C and the origin (food poisoning, veterinary or human clinical) of the *C. perfringens*. While the heat resistance of a majority of the strains was not significantly different ($p < 0.05$), one out of the three isolates of "1980s European: clinical", i.e., NB 16, exhibited significantly higher ($p < 0.05$) heat resistance as compared to one (NCTC 8239) out of the three "1950s European food-poisoning" and one (222) out of the two "1990s North American Veterinary" isolates (Table 1). This observation regarding the lack of correlation between the heat resistance at 100 °C and the origin of the *C. perfringens* isolates is not in agreement with those made by other researchers. Sarkar, Shivers, Sparks, Juneja, and McClane (2000) hypothesized that food poisoning is associated with chromosomal *cpe*

isolates and not with non-foodborne human gastrointestinal disease isolates carrying a plasmid *cpe* gene. Accordingly, the authors assessed the heat resistance of 13 isolates varying considerably with respect to their geographical origins and dates of isolation and reported that the food poisoning isolates exhibited increased heat resistance. The *D*-values at 90 or 100 °C for the *C. perfringens* chromosomal *cpe* isolates were significantly higher ($p < 0.05$) than the *D*-values of the *C. perfringens* isolates carrying a plasmid *cpe* gene. Thus, variations in heat resistance must be considered while designing acceptance limits on critical control points that ensure safety against *C. perfringens* spores in cooked foods. The *D*-values, expressed in minute, of *C. perfringens* spores heated in beef gravy at 100 °C ranged from 15.50 min (NCTC 8239 isolate) to 21.40 min (NB 16 isolate). Based on a r^2 value of >0.90 obtained using a linear regression, the thermal inactivation data could be fitted well to generate survivor curves. The analysis of covariance showed evidence of a significant treatment by strain interaction on the slopes of the suvivor curves. This was examined and turned out to be a consequence of a statistically significantly increase ($p < 0.05$) in *D*-values at 100 °C for 9 out of 10 isolates following heat shock (Table 1). The possible explanation for non-significant ($p < 0.05$) increase in *D*-value after heat shocking for the isolate NCTC 8238 remains unknown and needs to be explored. Compared to the control (no heat shock), the increase in heat resistance of *C. perfringens* spores after heat shocking ranged from 1.1-fold (NCTC 8238 isolate) to 1.5-fold (F 4969 isolate).

By selecting common test temperatures, the thermal inactivation data obtained in this study can be compared with those in the published literature on the heat resistance of *C. perfringens* spores. The thermal inactivation data reported in this study were, in general, consistent with those reported elsewhere. Bradshaw, Peeler, and Twedt (1977) reported *D*-values at 99 °C for *C. perfringens* spores suspended in commercial beef gravy ranged from 26 to 31.4 min. In a study by Heredia, Garcia, Luevanos, Labbe, and Garcia-Alvarado (1997), when heat resistance of *C. perfringens* spores was determined in double-distilled water using screw-capped tubes, the *D*-values at 85 and 95 °C of 55 and 24 min, respectively, were reported. Sarkar et al. (2000) reported *D*-values at 100 °C for 12 isolates of *C. perfringens* spores, carrying either chromosomal *cpe* gene or plasmid *cpe* gene, in DS medium ranged from 0.5 to 124 min. In other studies, *D*-values of 17.3 min in beef slurry (pH 5.5) and 23.2 min in turkey slurry (pH 6) at 99 °C for a spore cocktail of three *C. perfringens* strains were reported (Juneja & Majka, 1995; Juneja & Marmer, 1996). Differences in *D*-values obtained in our study and those reported by previous workers may be attributed to several factors. Sporulation media and temperature used for spore preparation, heating menstruum, recovery conditions including the composition and pH of the medium, the presence of inhibitors, temperature and time of incubation, and above all, the presence or absence of lysozyme in the recovery media, affect the calculated spore heat resistance (Foegeding & Busta, 1981; Juneja, 2000). Scott and Bernard (1982) suggested that there may be significant variations among strains, and the reported *D*-values by different investigators within the same strains.

While thermal processing guidelines are generally adequate for destruction of pathogens in foods, there may be conditions when the microorganism could become more heat resistant. Such conditions include environmental stresses occurring prior to cooking such as sublethal heat treatment or known as heat shocking conditions. Researchers have reported that such stressful environmental conditions experienced by pathogenic bacteria increase the resistance of a wide range of microorganisms, including *C. perfringens* (Heredia et al., 1997), *Bacillus stearothermophilus* (Beaman, Pankratz, & Gerhardt, 1988; Etoa & Michiels, 1988), *C. botulinum* (Appleyard & Gaze, 1993), and *Clostridium sporogenes* (Alcock, 1984), in foods to cooking. An increase in heat resistance of spores following heat shock obtained in this study was, in general, consistent with those reported in the literature. Heredia et al. (1997) heat shocked sporulating cells of *C. perfringens* at 50 °C for 30 min and then determined the *D*-values at 85 or 90 °C. The authors reported that a sublethal heat shock increased the thermotolerance of *C. perfringens* spores by at least 1.7 to 1.9-fold.

An attempt was made to explain the *D*-value increases, following heat shock at 75 °C for 20 min, by the induced expression of key heat shock proteins (HSPs; Fig. 2A–C). Western immunoblot analyses of spore lysates established the presence of proteins with epitopic similarity to *E. coli* GroEL (Fig. 2B) and *B. subtilis* SspC (Fig. 2C). Unfortunately, the increased presence of these proteins within the spores was not evident as a result of heat shock conditions (Fig. 2B and C). These results further support the dormant nature of the spores and imply any changes in expressed proteins associated with spore synthesis need be completed prior to the release of free spores by the mother cell. Additionally, our results suggest that HSPs are not the contributing factors in the development of thermotolerance of *C. perfringens* spores.

C. perfringens presents a significant food safety hazard and is likely to cause food poisoning in consumers of foods which have not been adequately processed or improperly stored at some point from production to consumption. The food safety implications of an increased heat resistance following heat shock are, obviously, of substantial practical importance to food processors. Accordingly, to guard against hazards and ensure adequate degree of protection against *C. per-*

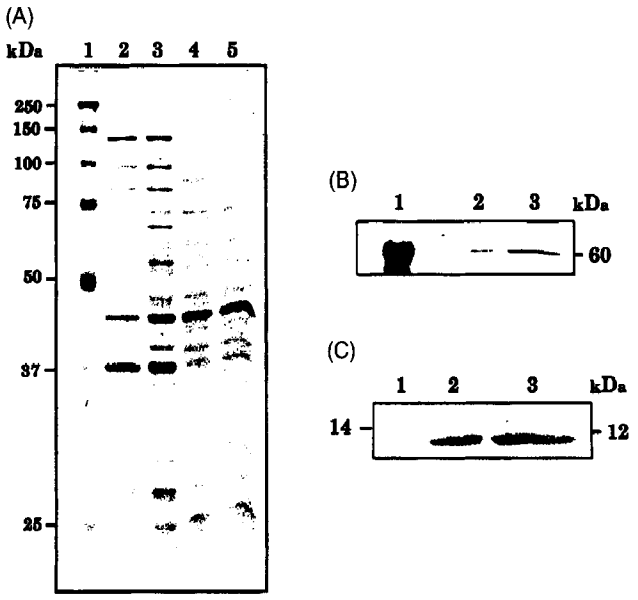

Fig. 2. (A) SDS-page comparison of *C. perfringens* strain NB16 vegetative cell and spore lysates. Twenty micrograms of total protein were loaded per lane. Lanes: 1, protein molecular weight standards; 2, vegetative cells non-heat shocked (NHS); 3, vegetative cells heat shocked at 48 °C for 10 min (HS); 4, spores non-heat shocked (NHS); and 5, spores heat shocked at 75 °C for 20 min (HS). Western immunoblots of spore lysates from *C. perfringens* strain NB16 were reacted with antisera raised against (B) *E. coli* GroEL and (C) *B. subtilis* SspC. Ten micrograms of total protein were loaded per lane. 2B lanes: 1, *E. coli* GroEL 60 kDa protein standard; 2, NB16 (NHS); and 3, NB16 (HS). 2C lanes: 1, 14 kDa protein molecular weight standard; 2, NB16 (NHS); and 3, NB16 (HS).

fringens spores in thermally processed foods, the heat shock response and induced thermotolerance of spores must be considered while designing thermal treatments for ready-to-eat foods, i.e., the time and temperature combinations for heating must be adequate to destroy contaminating *C. perfringens* spores exhibiting induced thermotolerance due to an exposure to stressful environmental conditions. Sufficient evidence exists to document that the spores can survive various meat cooking procedures (Craven, 1980; Wright-Rudolph, Walker, & Parrish, 1986). Under the conditions described in the current study, findings that the increased heat resistance of the spores can be as much as 1.5-fold further heightens the food safety concerns. Based on the thermal-death-time values and the phenomenon of heat shock response and induced thermotolerance of *C. perfringens* spores assessed in this study, heating time at 100 °C for contaminated beef gravy should be increased from 128.4 to 168.4 min; this is based on the argument that thermal treatments must be designed to achieve a 6-*D* process for *C. perfringens* spores. The data should alert food processors to take into account prior environmental conditions, and then design adequate thermal regimes to eliminate *C. perfringens* spores in thermally processed foods.

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